CIRCULAR RNA BTG2 INHIBITS THE PROLIFERATION AND METASTASIS OF ESOPHAGEAL SQUAMOUS CARCINOMA VIA MIR-92B-3P

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ABSTRACT

Introduction: *To explore the expression of CircBTG2 in esophageal squamous cancer (ESCC) and investigate the effect of CircBTG2 on the proliferation and invasion of ESCC.*

Materials and methods*: The medical records and pathological tissue of 33 ESCC patients treated in Lianshui County People's Hospital from January 2019 to April 2020 were collected and analyzed. The expression of CircBTG2 in normal esophageal mucosa and ESCC was detected by qRT-PCR. CircBTG2 overexpression plasmid was transfected into KYSE150 cells, an ESCC cell line, then cell proliferation was detected by CCK-8. Wound healing analysis, Transwell chamber invasion assay and clone formation experiment were performed to detect the metastatic ability of KYSE150 cells. The Luciferase reporter assay was used to detect whether CircBTG2 sponge miR-92b-3p. The expression levels of Kruppel like factor 4 (KLF4) and desmocollin 2 (DSC2) were detected by Western blot.*

Results*: The expression level of CircBTG2 in ESCC (2.994±0.927) was significantly lower than that in normal esophageal mucosa (4.76±1.503). After transfection of CircBTG2 overexpression plasmid into ESCC cell line KYSE150, the proliferation of CircBTG2 overexpression KYSE150 cells was significantly inhibited at 72h and 96h compared with the untransfected cells (t=2.364, 5.199; P=0.046, 0.001). In addition, the lateral migration, vertical invasion and colony formation of CircBTG2 overexpression KYSE150 cells were markedly inhibited. The results of luciferase assay showed that the fluorescence intensity of KYSE150 cells was significantly reduced after co-transfection of CircBTG2 overexpressing plasmid and miR-92b-3p mimics, suggesting that CircBTG2 has the ability to sponge miR-92b-3p. The results of Western blot demonstrated that the expression of KLF4 and DSC2 significantly increased after CircBTG2 overexpression (t=5.335, 6.688; P=0.006, 0.003).*

Conclusion*: In ESCC, the expression of CircBTG2 was significantly decreased. CircBTG2 promoted the expression of KLF4 and DSC2 via sponging miR-92B-3p, thereby inhibiting the proliferation and metastasis of ESCC. Therefore, CircBTG2 has potential as a diagnostic marker and therapeutic target for ESCC.*

Keywords: *CircBTG2, miR-92b-3p, esophageal squamous cancer, tumor metastasis.*

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Introduction

Esophageal cancer (EC) usually occurs in the mid-thoracic esophagus, and its annual number of new cases and deaths ranks ninth and sixth in the world's malignant tumours, respectively[(1)]. Esophageal squamous cell carcinoma (ESCC) makes up more than 70% of the EC in China. Currently, the clinical treatment of the ESCC is mainly surgical resection, complemented by chemotherapy or radiotherapy, but because of tumor metastases and other reasons, her five-year survival rate is only about 15%[(2)]. The onset of ESCC is insidious, and there is only foreign body sensation when swallowing at the early stage of the disease, which is easy to ignore by patients. At the same time, due to the lack of markers for early diagnosis of ESCC, early diagnosis of ESCC is challenging. Moreover, ESCC is very aggressive, and most patients diagnosed in the intermediate and late stages of the disease have tumor metastases, leading to treatment failure. The pathogenesis of the ESCC is still unclear, and it is

generally thought that its pathogenesis is related to factors such as heredity, unhealthy eating habits, smoking. With the development of high-speed gene sequencing technology and genetic chip technology, a large number of abnormal expression genes have been found in ESCC[3,4].

In recent years, studies have revealed that non-coding RNA plays a regulatory role in the development and development of the ESCC[5]. The circular RNA (circular RNA, CircRNA), as a non-coding RNA type, is predominantly located in the cytoplasm and has the function of regulating the expression of microRNA (miRNA). CircRNA forms a closed loop structure by trans-splicing, which is not readily degraded by RNase, and is relatively stable in cells, which has the potential for biomarkers[6-8]. Recent studies have pointed to the role of CircRNA as a diagnostic marker of tumour and therapeutic target in a variety of malignant tumours[9,10]. Our previous study showed that B cell translocation gene 2 (BTG2) was low expressed in ESCC, and its expression was significantly correlated with N stage and clinical stage of ESCC. Compared to patients with low BTG2 expression, patients with strong BTG2 expression have low radiation resistance and a better prognosis[11]. At the same time, Li et al. Found that circular RNA BTG2 (Circular RNA BTG2, CircBTG2), a "by-product" of BTG2, can compete with BMI-1 for binding to miR-330-3p, and is related to the occurrence and development of prostate cancer[12]. However, the expression and biological function of CircBTG2 in ESCC are still unknown. This study will analyze the expression of CircBTG2 in ESCC and normal tissues, and preliminarily explore the regulatory effect of CircBTG2 on ESCC.

Materials and methods

Human ESCC tissue samples

Paraffin-based pathological data and tissue were retrospectively collected and analyzed for confirmed ESCC patients at Lianshui County People's Hospital Affiliated to Kangda College of Nanjing Medical University from January 2019 to April 2020.

Case selection criteria:

• Confirmation of the ESCC by the pathology department;

• Neither surgery nor radical chemotherapy patients received chemotherapy prior to taking samples;

• No history of blood-borne illness or infection prior to treatment

Case exclusion criteria:

• Incomplete clinical data;

• Recent history of blood transfusion

• No follow-up records;

• Serious infection or autoimmune illness.

A total of 33 patients took part in the study, of which 27 were men and 6 were women. This study was conducted on all patients and approved by the Ethics Board of Lianshui County People's Hospital affiliated with Kangda College of Nanjing Medical University.

Main reagents

The human cell line ESCC KYSE150 was bought from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in a DMEM (Gibco) environment containing 10% bovine fetal serum (FBS, Gemini) and cultured in a humidified atmosphere containing 5% CO2 at 37°C. All cells used in this experiment have been in 10 generations. CircBTG2 overexpression vector pCD25-CircBTG2 and dual luciferase reporter gene vector psiCHECK2-CircBTG2 were synthesized by Guangzhou Jisai Biotechnology Co., Ltd. miR-92b-3p mimics and controls were purchased from Guangzhou Ruibo Technology Co., Ltd. The antibody for Western blotting was purchased from protein Tech (Wuhan Sanying).

CircBTG2 expression detection

The expression of CircBTG2 in normal esophageal mucosal tissue, ESCC tissue and KYSE150 cells was analyzed using real-time fluorescent quantitative PCR (qRT-PCR). TRIzol (Ambion) was used to extract total RNA from tissues and cells. The upstream primer of rcBTG2 is 5'-ACAGCTTATGGACAAATG-3', and the downstream primer is 5'-GGAAGTGCGGGTCCACAAGACAGCG-3'; the upstream primer of β-actin is 5'-AGGTCATCACTATTGGCAACGA-3', and the downstream primer is 5'-CACTTCATGATGGAATTGAATGTAGTT-3'.

CCK-8 Assay

Cell proliferation was investigated with the Cell Counting Kit-8 (CCK-8). The cells were stocked at a density of 3000 cells per well and 100 μl of medium per well in 96 well plates. The cells were grown at 37°C in a temperature-controlled incubator. After cultivation for 24h, 48h ,72h and 96h, 10 μl of CCK-

8 reagents (CCK-8, beyotime) were added, and absorption at 450nm was measured after 2h using a microreader.

Scratch assay experiments

Inoculate the cells into a six-well patch. When the cell monolayer covers the underside of the six-well plate, discard the middle, use a 1ml pipette to make a "wound" between the cells and wash them with PBS three times, then add the medium again. After incubation 24h, observed "injury" scarring, registered and photographed under an inverted microscope.

Test of cellular invasion

After the cells were systematically digested, they were resuspended in a DMEM medium containing 1% FBS, and 100 μl of the cell suspension was inoculated in the upper chamber. Add 600 μl of DMEM culture medium containing 10% FBS in the lower chamber. Place it in a 37°C steady-state incubator for cultivation. After 24 hours, carefully wipe the cells that have not migrated to the upper chamber and place the chamber into methanol for uptake. After fixing, color with 0.1% crystalline violet solution for 20 minutes. Remove the polycarbonate film after discolouration, watch and take photos under a microscope after mounting the film.

Colonies-formation assay

Add 500 cells to each well of the six-well slab and place in a constant temperature incubator at 37°C for 7 to 10 days. Observe cell morphology under a reverse microscope, and finish culture when a group of clear cells can be observed. Dispose of the middle and wash the cells using PBS. Add 2 ml of anhydrous methanol to each well and set the cells in a refrigerator at 4°C for 15 minutes. Dispose of the anhydrous methanol and clean the cells using PBS. Add 2 ml of the hematoxylin staining solution to each hole, after dyeing for 15 minutes, slowly wash the floating colour with running water. Take photographs and record them once the cells are naturally dried.

Western blotting assay

Expression levels for Kruppel factor 4 (KLF4) and desmocolline 2 (dsc2) were detected by Western blot. The total protein within the KYSE150 cells was extracted and measured. Cell lysates were loaded onto the SDS/polyacrylamide gel for electrophoresis and transferred over the PVDF membranes. Subsequently, PVDF membranes were clogged with 5% skim milk for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C. After washing with TBST for 3 times and incubated with a second antibody at ambient temperature for an hour. Finally, the membranes were visualised with the ECL reagent (EMD Millipore). ImageJ (version 1.48; National Institutes of Health) has been used for densitometry.

Dual-luciferase reporter assay

Two types of CircBTG2 [Wild (WT) or Mutant (MUT)] fragments were inserted into the psiCHECK2-CircBTG2 vector (Genesee, China) to obtain the CircBTG2-WT or -MUT declarant. KESY150 cells were co-transfected with miR-92b-3p mimics and CircBTG2-WT or -MUT by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 hours of transfection, the Dual-Luciferase Reporter Assay System (Promega Corporation) was used to evaluate luciferase activity.

Statistical Analysis

All statistical analyses were performed in SPSS 22.0 and Graphpad Prism 5.0. The data are expressed as mean ± standard deviation. Gene expression using Median (interquartile range). The comparison between the two groups was carried out by means of the double-sided t-test. One-way analysis of variance was used to compare differences across three or more groups, with difference considered statistically significant when the P-value was less than 0.05.

Results

The expression of CircBTG2 in ESCC tissues was significantly lower

First, we selected 13 normal esophageal mucosal cases and 33 ESCC cases. CircBTG2 expression has been detected by qRT-PCR. As shown in Figure 1A, the expression level of CircBTG2 in normal esophageal mucosa tissue is 4.76 ± 1.503; the expression level in ESCC tissue is 2.994 ± 0.927. Compared with the two, the difference was statistically significant (t=3.772, P=0.001).

CircBTG2 inhibits ESCC cell proliferation

To determine if CircBTG2 inhibits the proliferation of ESCC, the over-expression plasmid CircBTG2 was introduced into the KYSE150 cell line (Figure 2A). The results of the CCK-8 experiment

showed that after the transfer of the over-expression plasmid CircBTG2, KYSE150 cell proliferation in the CircBTG2 group was significantly inhibited at 72 h and 96 h compared to the untreated group and empty plasma group (t= 2.364, 5.199, P=0.046, 0.001, Figure 2B). At 48 hours, there was no significant difference in cellular proliferation in each group.

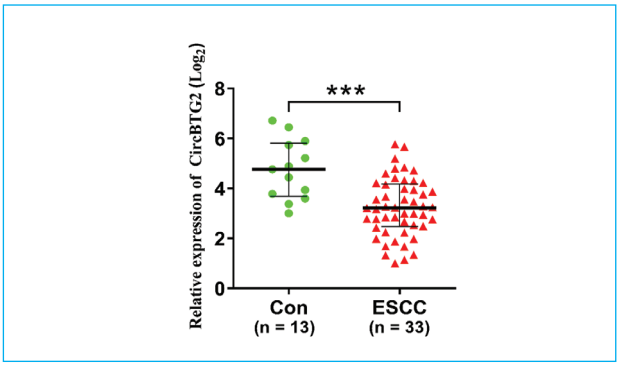

Figure 1: The expression of CircBTG2 in normal esophageal mucosa was significantly higher than that in ESCC. ***P < 0.001.

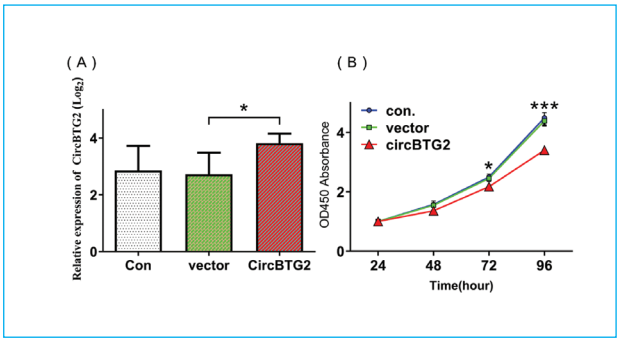

Figure 2: CircBTG2 inhibits the proliferation of ESCC cells. (**A**) Following transfection with CircBTG2 overexpression plasmids, the expression level of CircBTG2 in the untreated group, the empty plasmid group and the CircBTG2 overexpression group; (**B**) CCK-8 method for detecting cellular proliferation in all three groups at 24, 48, 72 and 96 hours. Compared with the untransfected group, *: P < 0.05, ***: P < 0.001.

CircBTG2 inhibits ESCC metastasis

The primary reason for treatment failure is tumour metastasis. Having confirmed that CircBTG2 can inhibit the proliferation of the ESCC, this section will further examine whether CircBTG2 can inhibit the metastasis of the ESCC. A wound healing test, a cell invasion test and a colony development test were used to detect KYSE150 cells. As shown in Figure 3, overexpression of CircBTG2 may significantly slow the healing of the KYSE150 "wound" cell, and has the ability to inhibit lateral migration of tumours (Figure 3A). Cell invasion experiments show that overexpression of CircBTG2 can significantly inhibit migration of KYSE150 cells to the polycarbonate membrane, and has the capacity to inhibit longitudinal tumour invasion(Figure 3B). The results of colony formation experiments showed that compared with the untransfected group and the empty plasmid group, the cell populations in the CircBTG2 overexpression group were significantly reduced, and CircBTG2 overexpression could inhibit the formation of cell colonies by single tumor cells(Figure 3C).

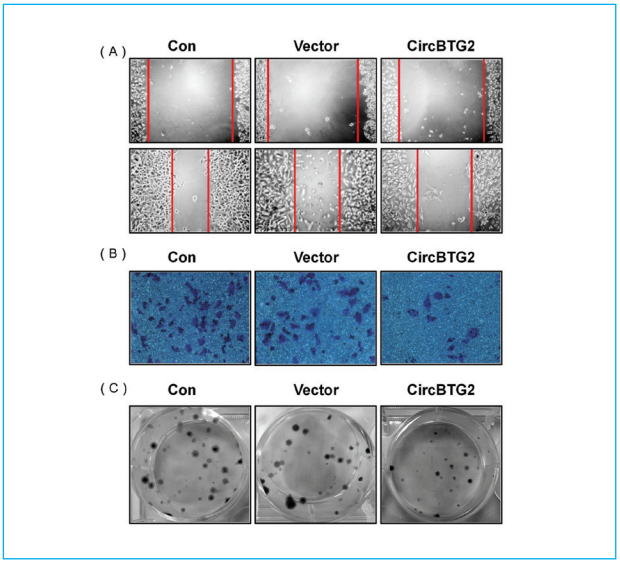

Figure 3: CircBTG2 inhibits ESCC metastasis and invasion. (**A**) Scratch test; (**B**) cell invasion test. (**C**) Colony formation experiment.

CircBTG2 interferes with the expression of KLF4 and DSC2 in ESCC

In the earlier study, we confirmed that miR-92b-3p can play a role in promoting the progress of the ESCC by removing the expression of KLF4 and DSC2[13]. To investigate if CircBTG2 can adsorb miR-92b-3p, we first used the reporter dual-luciferase gene to analyse if CircBTG2 can bind to miR-92b-3p. The detection of dual luciferase reporter gene system showed that the relative fluorescence of miRNA-92b-3p mimic added mutant vector group (Mut-CircBTG2) and control mimic + mutant vector group (Mut-CircBTG2) was compared (1.00 ± 0.14 vs. 1.03 ± 0.07), the difference was not statistically significant (P > 0.05)(Figure 4A). The relative fluorescence intensity of miR-92b-3p mimic + wild-type carrier group (wild-CircBTG2) is lower than that of control mimic + wild-type carrier group (wild-CircBTG2), and the difference is statistically significant (1.07 ± 0.23 vs. 0.61 ± 0.10, P = 0.033),

the results suggest that CircBTG2 has a binding site for miR-92b-3p. Furthermore, the results of western blot experiments showed that the protein expression of KLF4 and DCS2 in the CircBTG2 overexpression group was significantly increased compared with the untransfected group, and the difference was statistically significant (t=5.335, 6.688, P=0.0059, 0.0026) (Figure 4B).

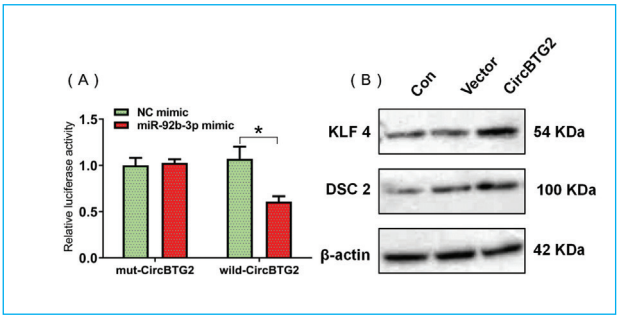

Figure 4: CircBTG2 promotes the expression of KLF4 and DSC2 in ESCC by binding miR-92b-3p. (A) Double luciferase reporter gene analysis; (B) Western blotting method to detect the expression of KLF4 and DSC2.

Discussion

In this study, we first analyzed the expression of CircBTG2 in normal esophageal mucosal tissues and ESCC tissues, and found that the expression of CircBTG2 in ESCC tissues was significantly lower than its expression in normal esophageal mucosal tissues (Figure 1A). After over-expression of CircBTG2 on the ESCC KYSE150 cell line, we found that KYSE150 cell proliferation was significantly inhibited at 72h and 96h (Figure 2B). Further research revealed that its horizontal migration capacity, vertical invasion capacity, and the ability of a single tumour cell to form colonies were inhibited by over-expression of CircBTG2 (Figure 3). Analysis of the double luciferase reporter gene revealed that CircBTG2 has the ability to bind to miR-92b-3p (Figure 4A). Western blot experiments results showed that in the CircBTG2 overexpression group, the expressions of KLF4 and DSC2 were significantly increased (Figure 4B). These results suggest that circbtg2 can promote the expression of KLF4 and DSC2 by binding miR-92b-3p, and inhibit ESCC proliferation and metastases.

CircRNA is involved in the regulation of various physiological and pathological processes in the body, such as the regulation of gene transcription, alternative splicing and the RNA-binding protein[8,14]. In particular, CircRNA has a large number of miRNA binding sites and acts as a inhibitor of the biological effects of miRNAs, and is recognized as a "sponge" miRNA[15]. Human CircBTG2, Hsa_Circ_0016068, stands at chr1:203274663-203278729. It consists of the circularization of the first and second exons of the BTG2 gene. It is the only BTG2-produced CircRNA in the human body (16). There is no report about the regulation of the mine by CircBTG2, but we have discovered through the steps of bioinformatic prediction software that CircBTG2 is very likely to link miR-92b-3p.

Consequently, in this study, we used the double luciferase reporter gene system to detect if CircBTG2 can bind to miR-92b-3p. As shown in Figure 4a, the fluorescence brightness of KYSE150 cells Co transfected with CircBTG2 overexpression plasmid and miR-92b-3p mimic was significantly lower than that of cells transfected with only CircBTG2 overexpression plasmid, which indicated that CircBTG2 had the "sponge" effect of binding with miR-92b-3p. However, the CircBTG2 and miR-92b-3p linking site is still unclear and further research is necessary.

Mirna plays a critical role in the development and development of tumours[17,18]. In recent years, studies have shown that miR-92b-3p plays a role in promoting tumour progression in a variety of tumours, including ESCC, prostate cancer, colorectal cancer and gastric cancer[13,19,20]. Wang found that compared with normal tissues, miR-92b-3p showed a significant overexpression trend in prostate cancer, and its overexpression was closely related to prostate cancer metastasis, suggesting a poor prognosis[21]. Similarly, our previous study found that in the ESCC, miR-92b-3p expression was significantly increased and its expression was significantly related to the clinical stage and stage N of the ESCC[13]. In addition, ESCC patients with elevated miR-92b-3p expression have a shorter survival period and a worse prognosis. Further studies have found that miR-92b-3p can inhibit the expression of the two proteins by binding to KLF4 and DSC2 mRNA, and play a role in promoting ESCC proliferation and metastasis. In this study, we have confirmed through dual luciferase reporter gene analysis and western blot experiments that CircBTG2 not only has the ability to bind to miR-92b-3p, but also promotes the expression of KLF4 and DSC2 proteins.

In conclusion, this study found that, on the one hand, the expression of CircBTG2 in ESCC tissues is significantly lower than that in normal esophageal mucosa tissues, and CircBTG2 has a closed loop structure, which makes it stable in the cytoplasm.

As a result, CircBTG2 can be used as a diagnostic marker for ESCC. On the other hand, the CircBTG2 can promote the expression of KLF4 and DSC2 by linking miR-92b-3p, thus inhibiting the proliferation and metastasis of the ESCC. This suggests that CircBTG2 has the potential to become a therapeutic target for ESCC, but its specific mechanism of ESCC inhibition remains to be studied.

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Author Contributions

Juan Pu, Wanpeng Wang conceived and designed the research methods. Wei Wang, Ran Yu, and Yao Xiao collected the data; Xiaolu Lin and Suqin Zhou analysed the data; Suqin Zhou, and Haoyu Chen wrote the original draft. Wei Wang, Ran Yu, Yao Xiao reviewed and edited the manuscript. All authors read and approved the final manuscript.

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