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A METHOD BY WHICH circUBR1 CONTROLS THE PROLIFERATION, MIGRATION, AND INVASION OF HSC3 CELLS

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Abstract

This work investigates the effects of circUBR1 and the underlying mechanisms on the growth, migration and penetration of HSC3 cells derived from oral squamous carcinoma. The expression levels of circUBR1, miR-216a-3p and HSC3 were assessed in tissues from oral squamous cell carcinoma (OSCC) and adjacent tissues.

Based on the transfection targets, the cells were divided into four groups: si-NC, si-circUBR1, miR-NC, miR-216a-3p, si-circUBR1+anti-miR-NC and si-circUBR1+anti-miR-216a-3p. The cell viability, scratch healing rate, MMP-2 and MMP-9 expression, number of cell clones formed, and number of invasion cells were significantly lower in the miR-216a-3p group compared to the miR-NC group ($p < 0.05$).

Moreover, when comparing the si-circUBR1+anti-miR-NC group to the si-circUBR1+anti-miR-216a-3p group, the cell viability, scratch healing rate, MMP-2 and MMP-9 expression, number of cell clones formed, and number of invasion cells were significantly higher in the si-circUBR1+anti-miR-216a-3p group ($p < 0.05$). This suggests that disrupting circUBR1 expression leads to up-regulation of miR-216a-3p, resulting in reduced proliferation, clone formation, migration, and invasion of OSCC cells.

Key words: OSCC, circUBR1, miR-216a-3p, cell proliferation, circular RNAs

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Introduction. The 5-year survival rate for patients with early OSCC is more than 70%, while it is lower than 20% for patients with advanced OSCC. The occurrence and progression of OSCC are closely correlated with the inactivation of oncogenes or the activation of tumour suppressor genes [1, 2]. Exonucleases do not break down the closed circular structure of circular RNAs (circRNA). Studies have demonstrated that circRNAs are high in microRNA (miRNA) binding sites and can act as a sponge to adsorb miRNAs, inhibiting their inhibitory effects on target gene expression and ultimately contributing to OSCC and its progression [3, 4].

Because lung cancer cells had significantly higher levels of circUBR1 expression, it was possible to decrease lung cancer cell growth, migration, and invasion as well as increase cell death by driving circUBR1 expression to decline [5]. MiR-216a-3p can limit the proliferation, clone formation, and invasion of cervical cancer cells, as well as its expression has been demonstrated to be reduced in cervical cancer tissues and cells in studies [6]. It is yet unclear how circUBR1iR-216a-3p affects the biological operation of OSCC cells. In this study, we focused primarily on circUBR1's impacts and potential mechanisms on biological activities such as OSCC cell invasion, migration, and proliferation.

Materials and methods. ***Experimental grouping.*** When HSC3 cells were 70% confluent, transfections were carried out. Ten percent neonatal bovine serum and 90% DMEM made up the HSC3 cell complete media. DMEM medium, newborn bovine serum, trypsin, and Trizol reagent were bought from Wuhan Shanen Biotechnology Co., Ltd. In 250 L of media without serum, si-NC, si-circUBR1, miR-NC, miR-216a-3p mimics, anti-miR-NC, and anti-miR-216a-3p were diluted and incubated for 5 and 20 min, respectively, at room temperature. After the transfectant has been diluted, it was thoroughly combined with the diluted transfection reagent. For 6 h, the combination was introduced to the culture of HSC3 cells. It was added to the entire medium and cultured for 48 h after discarding the culture solution. The si-NC, si-circUBR1, miR-NC, miR-216a-3p mimics, anti-miR-NC, anti-miR-216a-3p, pcDNA, and pcDNA-circUBR1 were purchased from Wuhan Miaoling Biotechnology Co., Ltd.

Groups for the experiment. Four groups of HSC3 cells that had been transfected using the above-said methods were created: si-NC, si-circUBR1, miR-NC, and miR-216a-3p. Human OSCC cells and HSC3 were bought from Wuhan Procell Life Science & Technology Co., Ltd.

Si-circUBR1, anti-miR-NC, si-circUBR1, and anti-miR-216a-3p were co-transfected into HSC3 cells using the transfection as mentioned earlier technique (referred to as si-circUBR1+anti-miR-NC group and si-circUBR1+anti-miR-216a-3p group, respectively). OSCC tissues, para-carcinoma tissues, and cells from each group had their total RNA extracted to examine the expression of circUBR1 and miR-216a-3p. CircUbBR1 and miR-216a-3p expression levels were evaluated by qRT-PCR.

Reaction conditions. The relative expression of miR-216a-3p and cir-

cUBR1 was assessed using the 2-Ct technique. Forty cycles of pre-denaturation were carried out at 95 °C for 2 min, 30 s of 95 °C denaturation, 30 s of 60 °C annealing, and 30 s of 72 °C extension.

Cell proliferation assay. CCK-8 reagent, Transwell chamber, and Matrigel were purchased from Shanghai Beyotime Inc. Ten litres of CCK-8 solution was used to seed HSC3 cells into each well, and they were then left to incubate for 2 h. A microplate reader was used to determine the optical density (OD) value at 450 nm in order to evaluate the viability of cell proliferation [6].

Cell clone formation, number detection. The number of cell clones formed was calculated using a plate clone creation test. Each set of HSC3 cells was grown in the incubator until a macroscopic cell clonogenic mass appeared. After washing and removing the supernatant, the cell staining was done for 15 min with 1% crystal violet before being fixed for 20 min with methanol. It was photographed after being rinsed in filtered water, and the number of cell clones formed was also noted [5].

Cell scratch healing rate assay. Each group's HSC3 cells were then grown in the incubator until they were fully developed. Using a pipette tip to scratch the cell monolayer, a scratch assay was used to measure the cell scratch healing rate (0 h at the moment). The cells spent an additional 24 h in the incubator. Under a microscope, the cell movement distance was seen. The cell scratch healing rate is equal to (0% scratch width – 100% scratch width after 24 h) scratch width [6, 7].

Cell invasion assay. There was a transwell test used. There were objects in the cave's upper room, matrigel, HSC3 cells, and complete media. Cells were treated with paraformaldehyde and rinsed after 48 h of culture. The addition of crystal violet staining allowed for the microscopic counting of the number of cell invasions [7].

Target relationship detection of circUBR1 with miR-216a-3p. MiR-NC or miR-216a-3p mimics were co-transfected into HSC3 cells to create WT-circUBR1 and MUT-circUBR1. After 2 h of culture, luciferase activity was discovered. In HSC3 cells, pcDNA, pcDNA-circUBR1, si-NC, and si-circUBR1 were transfected. After 48 h of culture, the relative expression of miR-216a-3p was identified [7].

Detection of MMP-2 and MMP-9 protein expression. Total protein was collected from HSC3 cells of each group, and MMP-2 and MMP-9 protein expression was assessed by Western blot analysis. The dual luciferase reporter vector and its activity assay kit were bought from Promega, USA; rabbit anti-human MMP-2, MMP-9, and GAPDH primary antibodies were bought from Wuhan Boster Biological Technology, Ltd. The ImageJ application was used to analyze the grayscale values of each band to ascertain the relative expression levels of the MMP-2 and MMP-9 proteins [6, 7].

Statistical analysis. Several groups were compared simultaneously using one-way ANOVA (snk-q test was used to compare two groups). The data were

Table 1

Comparison of the expression of circUBR1 and miR-216a-3p in para-carcinoma tissues and OSCC tissues ['x', $n = 49$]

Grouping	circUBR1	miR-216a-3p
Para-carcinoma tissues	1.00 ± 0.09	1.00 ± 0.07
OSCC tissues	3.87 ± 0.30	0.29 ± 0.05
t	64.142	55.775
P	0.000	0.000

Effects of interfering with circUBR1 expression on HSC3 cell proliferation [x ± s, $n = 9$]

Grouping	circUBR1	OD [450 nm]	Cell clone formation number [s]
si-NC	1.00 ± 0.00	0.97 ± 0.07	84.68 ± 5.65
si-circUBR1	0.40 ± 0.05	0.44 ± 0.04	37.68 ± 4.22
t	36.000	19.722	19.994
P	0.000	0.000	0.000

examined using SPSS 21.0. The metrology data were represented as ('xs), and the means of the t-test was used to compare data from two groups. Differences were considered statistically significant if $p < 0.05$.

Results. In contrast to para-carcinoma tissues, which are demonstrated in Table 1 to have significantly higher expression of circUBR1, OSCC tissues had much lower expression of miR-216a-3p.

In comparison to the si-NC group, the si-circUBR1 group's cell survival and the number of clones formed were considerably lower ($p < 0.05$). According to Table 2, the si-circUBR1 group's scratch healing rate, MMP-2 and MMP-9 expression, and cell invasion were noticeably lower than those of the si-NC group ($p < 0.05$) (Fig. 1, Table 2). Table 2 shows that the nucleotide sequences of CircUBR1 were complementary to miR-216a-3p (Fig. 2). Over-expression of miR-216a-3p significantly ($p < 0.05$) reduced the luciferase activity of the wild-type vector WT-circUBR1, but did not affect the mutant vector MUT-circUBR1. While si-circUBR1 expression was significantly higher in the si-circUBR1 group compared to the si-NC group, miR-216a-3p levels were markedly lower in the pcDNA-circUBR1 group compared to the pcDNA group ($p < 0.05$).

Comparing the miR-216a-3p group to the miR-NC group, the miR-216a-3p group displayed significantly lower levels of cell survival, scratch healing rate, expression of MMP-2 and MMP-9 protein, cell clone creation number, and invasion cell number ($p < 0.05$) (Fig. 1, Table 3). Table 3 also demonstrates that the si-circUBR1+anti-miR-216a-3p group considerably outperformed the si-circUBR1+anti-miR-NC group in terms of cell viability, scratch healing rate, protein expression of MMP-2 and MMP-9, cell clone formation number, and invasive cells ($p < 0.05$).

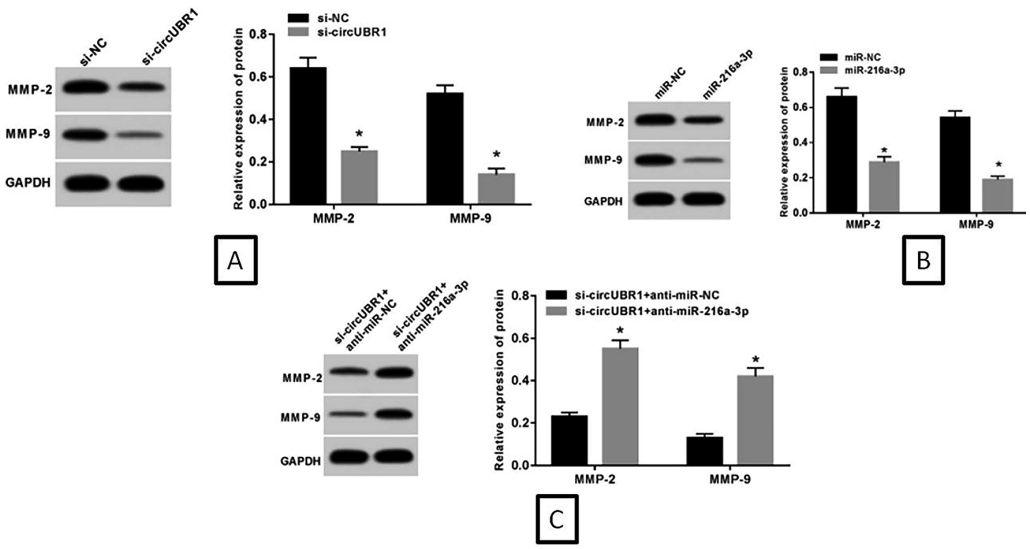

Fig. 1. A. The impact of suppressing circUBR1 expression on the expression of MMP-2 and MMP-9 proteins in HSC3 cells; B. Effects of miR-216a-3p expression being downregulated on MMP-2 and MMP-9 protein expression in HSC3 cells; C. Inhibiting circUBR1 expression has the opposite effect on MMP-2 and MMP-9 protein expression in HSC3 cells as does suppressing miR-216a-3p expression

Discussion. CircRNAs may serve as tumour molecular markers or therapeutic targets since they are intimately linked to the development of malignancies and can control target gene expression by adsorbing miRNAs and circRNAs [7,8]. CircRNAs are abnormally expressed in OSCC and can change the biological activity of OSCC cells by controlling the expression of miRNA and mRNA [9–11]. Breast cancer tissues and cells express circUBR1 at a much higher level than healthy tissues and cells. Reduced circUBR1 expression has increased cell apoptosis and reduced breast cancer cell growth and metastasis [12].

A pertinent investigation on the expression and mechanism of circUBR1 in OSCC, both domestically and internationally, is still lacking. In this investigation, we found that OSCC patients expressed circUBR1. In contrast to para-carcinoma tissues, we discovered that OSCC tissues had significantly greater levels of circUBR1 expression. Further cell research revealed that suppressing the expression of circUBR1 could lower the viability and proliferation of OSCC cells, indicating that circUBR1 may be strongly linked to the pathogenesis of OSCC. Reduced circUBR1 expression may reduce the viability of cell growth.

The invasion and metastasis of many malignant tumours are closely related to MMP-2 and MMP-9, which can promote tumour cell movement and invasion [13,14]. In this study, we found that down-regulating circUBR1 expression significantly reduced the scratch healing rate, the MMP-2 and MMP-9 protein levels, and the number of invasion cells in OSCC cells, demonstrating that circUBR1 is connected to OSCC cell migration and invasion.

Table 2

Findings regarding circUBR1 expression on the amount of HSC3 cells, regulation and control of miR-216a-3p expression

Grouping	Scratch healing rate (%)	Cell invasion number(s)
si-NC	68.77 ± 5.57	125.25 ± 11.93
si-circUBR1	27.23 ± 3.58	52.36 ± 5.29
t	18.821	16.756
P	0.000	0.000

circUBR1 regulates miR-216a-3p expression

Grouping	WT-circUBR1	MUT-circUBR1
miR-NC	0.93 ± 0.06	0.97 ± 0.05
miR-216a-3p	0.34 ± 0.03	0.94 ± 0.05
t	26.386	1.273
P	0.000	0.221

circUBR1 controls the expression of miR-216a-3p

Grouping	miR-216a-3p
pcDNA	1.00 ± 0.00
pcDNA-circUBR1	$0.51 \pm 0.06^{*}$
si-NC	1.02 ± 0.06
si-circUBR1	$3.08 \pm 0.31^{\#}$
F	455.24
P	0.00

Note: When comparing the si-NC group to the pcDNA group, $^{*}p < 0.05$; when comparing the two, $^{\#}p < 0.05$

In comparison to normal tissues, lung cancer tissues and cells have considerably decreased levels of miR-216a-3p expression, which can negatively influence lung cancer cell growth, migration, and invasion as well as increase apoptosis to have anti-tumour effects [15]. The current investigation provided further evidence of target regulation between circUBR1 and miR-216a-3p. Additionally, circUBR1 might work as a miR-216a-3p sponge molecule to inhibit miR-216a-3p expression.

Our findings demonstrated that the expression of miR-216a-3p was reduced in OSCC tissues. MiR-216a-3p expression is significantly downregulated in colorectal cancer, whereas miR-216a-3p expression increases and inhibits disease progression [16]. Additionally, it was discovered that cholangiocarcinoma cells drastically reduced miR-216a-3p expression and that increased miR-216a-3p expression might prevent the biologically malignant behaviours of cholangiocarcinoma cells [17]. The increased miR-216a-3p expression may harm HSC3 cells' ability to proliferate, create clones, migrate, and invade. On the other hand, circUBR1 expression inhibition might undo the restriction on HSC3 cell proliferation and metastasis, suggesting that circUBR1 regulates miR-216a-3p expression to limit the OSCC cells' malignant biological activity.

T a b l e 3

Effects of increased miR-216a-3p expression on HSC3 cell motility, invasion, and proliferation ('x', $n = 9$); Inhibiting circUBR1 expression has the opposite effect on MMP-2 and MMP-9 protein expression in HSC3 cells

Grouping	miR-216a-3p	OD (450 nm)	Cell clone formation number (s)	Scratch healing rate (%)	Number of invasion cell (s)
miR-NC	1.00 ± 0.00	0.98 ± 0.07	87.94 ± 6.67	66.72 ± 6.09	127.47 ± 12.81
miR-216a-3p	$3.45 \pm 0.22^*$	$0.51 \pm 0.04^*$	$44.67 \pm 4.05^*$	$32.91 \pm 2.72^*$	$61.27 \pm 5.14^*$
t	33.40	17.48	16.63	15.20	16.56
P	0.00	0.00	0.00	0.00	0.00
si-circUBR1+anti-miR-NC	1.00 ± 0.00	0.43 ± 0.04	35.96 ± 3.14	26.44 ± 2.36	50.84 ± 4.14
si-circUBR1+anti-miR-216a-3p	$0.31 \pm 0.03^*$	$0.88 \pm 0.07^*$	$75.18 \pm 5.48^*$	$57.93 \pm 5.48^*$	$103.93 \pm 10.68^*$
t	69.00	16.74	18.62	15.83	13.90
P	0.00	0.00	0.00	0.00	0.00

Note: Compared with the miR-NC group, $^*p < 0.05$;
Notes: $^*p < 0.05$ versus si-circUBR1+anti-miR-NC group

In conclusion, OSCC tissues had considerably higher circUBR1 expression. While inhibition with circUBR1 expression could prevent OSCC cells from proliferating, cloning, migrating, and invading, miR-216a-3p expression was markedly downregulated. The underlying method involves enhancing miR-216a-3p expression to control OSCC cell growth.

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