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Original Research Article

Reticulocalbin-2 promotes the proliferation and migration of oral squamous cell carcinoma by regulating the EGFR-ERK pathway

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Abstract

Purpose: *To investigate the expression of reticulocalbin-2 (RCN2) in human oral squamous cell carcinoma (OSCC) tissues and cell lines and its effects on the proliferation and migration of OSCC cells.*
Methods: *Immunohistochemical (IHC) assays were performed to evaluate the expression of RCN2 in 30 histologically-confirmed OSCC patient tissues and adjacent tissues. Immunoblot assays were performed to examine the expression of RCN2 in an oral mucosal keratinocyte cell line and OSCC cell lines, while the effects of RCN2 on OSCC cell proliferation were assessed by CCK-8 and colony formation assays. The effects of RCN2 on OSCC cell motility were determined using wound closure and Transwell assays. Furthermore, the effect of RCN2 on EGFR/ERK pathway in OSCC cells was evaluated by immunoblot assay.*
Results: *Overexpression of RCN2 occurred in OSCC tissues and cells ($p < 0.01$). RCN2 also increased the proliferation of OSCC cells and stimulated the motility of OSCC cells in vitro ($p < 0.01$). These effects occurred as a result of the regulation of EGFR/ERK signaling pathway by RCN2.*
Conclusion: *RCN2 is a potential therapeutic target for OSCC treatment. However, further in vivo studies are required to validate these findings.*

Keywords: *Reticulocalbin-2 (RCN2), Oral squamous cell carcinoma (OSCC), Proliferation, Motility, EGFR/ERK pathway*

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a common type of cancer that affects the oral cavity, including the tongue, gingiva, mouth floor, and labial mucosa [1]. OSCC accounts for nearly 3% of tumors, with approximately 550,000 new cases worldwide every year [2,3]. Smoking and drinking alcohol are major risk factors for OSCC, though genetics may also contribute to the disease. To date, the main treatment for OSCC involves surgical resection accompanied by radiotherapy and chemotherapy; however, the mortality of OSCC patients remains high with survival less than 50 % [3]. In recent years, targeted therapy has shown promising

advantages, and identifying more effective targets for OSCC treatment is crucial [4]. Reticulocalbin (RCN) is a Ca^{2+}-binding protein family that functions in secretion-related pathways [5]. There is growing evidence that members of the RCN family may also play a role in tumorigenesis, tumor invasion, and drug resistance [6]. RCN mediates the proliferation, migration, and apoptosis of cancer cells in multiple types of cancers [7]. RCN2 is located in the endoplasmic reticulum and is upregulated in HCC and plays a key role in HCC cell proliferation and tumor growth. The expression of RCN2 is also upregulated in colorectal cancer tissues and correlates with tumor growth and proliferation [7]. RCN2 could interact with EGFR, and the depletion of RCN2 in HCC cells suppressed the activation of the EGFR-ERK pathway by suppressing EGF-mediated internalization of EGFR [8]. However, the role of RCN2 in OSCC remains undetermined. The Cancer Genome Atlas (TCGA) data show that RCN2 is highly expressed in squamous cell carcinomas of the head and neck. Therefore, RCN2 may affect the progression of OSCC. The effect of RCN2 in OSCC was investigated in this study.

EXPERIMENTAL

Antibodies and plasmids

The following primary antibodies were purchased from Abcam: anti-RCN2 (1:100 dilution and 1:500 dilution for IHC and immunoblot, respectively, ab104516), anti-EGFR (1:500 dilution, ab52894), anti-p-EGFR (1:1000 dilution, ab40815), anti-ERK (1:500 dilution, ab184699), anti-p-ERK (1:500 dilution, ab201015), and anti-GAPDH (1:3000 dilution, ab9485). NC (negative control) siRNAs and RCN2 siRNAs were purchased from RioBio. Plasmids, including the pcDNA3.1-vector and pcDNA3.1-control, were constructed in our laboratory.

Immunohistochemical (IHC) assays

Thirty histologically-confirmed tumor tissues and normal tissues were obtained from patients at The Central Hospital of Wuhan. All procedures in this study were approved and in accordance with the standards upheld by the Ethics Committee of The Central Hospital of Wuhan (approval no. 2020-002), and also followed the guidelines of World Medical Association Declaration of Helsinki and Ethical Principles for Medical Research involving human subjects [9]. Protein expression levels in OSCC tumor tissues and normal tissues were determined using IHC assays. Sections (5-μm thick) were fixed at room temperature using 4% paraformaldehyde (PFA) for 30 min. Fixed sections were dewaxed with xylene at 65 °C and rehydrated in a gradient ethanol buffer. Samples were immersed in citrate buffer (pH, 6.0) at 98 °C for 30 min, placed in a microwave for antigen retrieval at 20 °C, and blocked with 2 % BSA (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. Blocked sections were incubated with the indicated primary antibodies at 4 °C overnight and subsequently incubated with biotinylated secondary antibodies for 1.5 h. The chromogen substrate was then used.

The expression level of RCN2 was classified into four groups based on the staining intensity (0, negative; 1, low; 2, medium; and 3, high). The proportion of cells was divided as follows: 0, 0 % stained cells; 1,1-25 % stained cells; 2, 26-50 % stained cells; and 3, 51-100 % stained cells. The staining intensity was calculated as the staining intensity score × the score of the percentage of stained cells. Scores < 2 were considered weak staining, 2-3 moderate staining, and > 4 strong staining.

Cell culture and transfection

Human oral mucosal keratinocyte line HOK and OSCC cell lines CAL-27, OSC-4, and HSC-3 were purchased from ATCC and maintained in DMEM with 10% fetal bovine serum (FBS) at 37 °C in a 5 % CO_2 incubator. Negative control siRNAs and RCN2 siRNAs were purchased from RioBio. The plasmids, including pcDNA3.1 and pcDNA3.1-control, were constructed in our laboratory. Plasmids and siRNAs were transfected into OSCC cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific). Viral supernatants were harvested using ultracentrifugation (600 g, 5 min, 4 °C). A total of 1×10^5 T24 or 5637 cells were seeded into 6-well plates and 0.5 μg plasmid or 5 μL siRNA was used. Cells were transfected using 10 μL Lipofectamine® 3000 (Invitrogen, Thermo Fisher Scientific) in each well. Following incubation for 20 min at 20 °C, the transfection was completed. Transfection efficiency was measured using both reverse transcription-quantitative PCR (RT-qPCR) and immunoblot analysis after 48 h.

Immunoblot assay

Cell samples were lysed using RIPA buffer (9800; Cell Signaling). Protein samples from cell and tissue samples were separated by 8 % SDS-PAGE, transferred onto PVDF membranes for 2 h, and blocked with 5 % fat-free milk in TBST buffer for 2 h. All membranes were subsequently

treated with the indicated primary antibodies at 4 °C overnight. Membranes were treated with secondary antibodies for 1 h at room temperature. Blots were then measured. The housekeeping gene was GAPDH.

CCK-8 assay

OSCC cells were plated into 96-well plates and maintained for 48 h after the transfection of plasmids or siRNAs. Cells were incubated with CCK-8 for 4 h and absorbances were measured at 490 nm.

Colony formation assay

OSCC cells were re-seeded into 6-well plates at 1000 cells per well and maintained for approximately 2 weeks until colonies were formed. Colonies were fixed with PFA for 10 min and stained with 0.1 % crystal violet for 20 min. After staining, colonies were photographed and counted.

Scratch wound assay

CAL-27 cells transfected with the indicated plasmids were grown to confluency. Scratches were made with a 10 µL-pipette tip and wells were washed with PBS buffer. Serum-free culture medium was added to induce wound closure. Images were taken to analyze cell migration. Control groups were cells transfected with the control plasmids or siRNAs. Wound widths were measured at 0 h and 24 h to assess the migration capacity of OSCC cells.

Transwell assays

Cells transfected with the indicated plasmids were plated into the upper chamber of transwell chambers in serum-free culture medium. Complete culture medium was added to the bottom chambers to stimulate cell invasion. After culturing for 24 h, cells in the upper chamber were removed, and the remaining cells were fixed, stained with 0.2 % crystal violet, and quantified using a microscope (Zeiss, Germany).

Statistics

GraphPad 5.0 software was used for statistical analyses. Three repeats were used in the *in vitro* assays. Data are presented as mean ± SEM. Student's t-tests were used for comparison. $p < 0.05$ was considered significant.

RESULTS

RCN2 was overexpressed in OSCC tissues and cells

To investigate the effects of RCN2 on OSCC, the expression of RCN2 was detected in human OSCC tissues and adjacent non-tumor tissues. Bioinformatic analysis was performed, and transcripts per million of RCN2 in tumor tissues were compared to normal tissues for calculation (Figure 1 A). RT-qPCR and immunoblot assays indicated that RCN2 was upregulated in human OSCC tissues (Figure 1 B and C). IHC assays indicated that RCN2 was overexpressed in 30 of the OSCC tissues as compared to the non-tumor tissues (Figure 1 D). Two representative stained images of OSCC and adjacent tissues are shown in Figure 1 D.

The clinical features of patients with low or high RCN2 expression were investigated. Interestingly, RCN2 expression correlated with lymph node metastasis ($p = 0.036$) and TNM stage ($p = 0.029$) of OSCC patients (Table 1). However, there were no significant correlations between RCN2 expression and patient age, sex, or differentiation grade (Table 1).

RCN2 expression levels were determined in the oral mucosal keratinocyte cell line HOK and OSCC cell lines CAL-27, OSC-4, and HSC-3. Immunoblot assays indicated that RCN2 was overexpressed in the OSCC cell lines compared to the HOK cell line (Figure 1E). Therefore, RCN2 was overexpressed in OSCC tissues and cells.

RCN2 promotes the proliferation of OSCC cells

Since RCN2 expression was upregulated in OSCC tissues and cells, the role of RCN2 was investigated in OSCC cells. RCN2 siRNAs and pcDNA3.1-RCN2 plasmids were transfected into CAL-27 cells to alter the expression of RCN2. Immunoblot assays indicated that RCN2 expression decreased after siRNA transfection and increased after transfection with pcDNA3.1-RCN2 plasmid in OSCC cells (Figure 2 A).

RCN2 depletion suppressed the proliferation of CAL-27 cells as indicated by CCK-8 assays (Figure 2 B). Overexpression of RCN2 promoted cell proliferation (Figure 2 B).

Table 1: RCN2 expression in samples from OSCC patients and its relationship with clinical pathological parameters

Characteristic	Total (n=30)	High expression (n = 15)	Low expression (n = 15)	*P*-value
Sex				0.715
Male	14	8	6	
Female	16	7	9	
Age				0.710
<60	12	5	7	
≥60	18	10	8	
Lymph node metastasis				0.009
Yes	14	11	3	
No	16	4	12	
TNM stage				0.003
I/II	17	4	13	
III/IIII	13	11	2	
Differentiation grade				0.653
Well	10	4	6	
Moderate	9	4	5	
Poor	11	7	4	

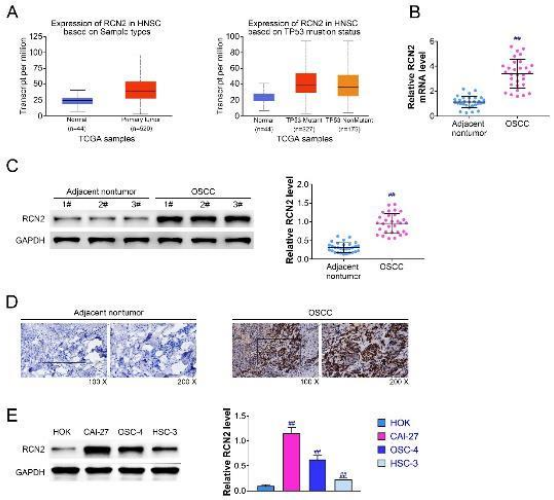

Figure 1: Overexpression of RCN2 in OSCC tissues and cells. (A) Data from the TCGA database showed RCN2 expression (transcripts per million) in normal and primary tumor tissues. (B) mRNA levels of RCN2 in OSCC and adjacent non-tumor tissues. (C) RCN2 expression in three representative non-tumor and OSCC tissues was confirmed using immunoblots. (D) Expression of RCN2 in two representative OSCC tumor tissues and adjacent normal epithelial tissues. (E) Expression of RCN2 in the OSCC cell lines CAL-27, OSC-4, and HSC-3. Data are presented as mean ± SEM (## $p < 0.01$)

Similarly, colony formation assays showed that RCN2 depletion inhibited CAL-27 cell proliferation and RCN2 overexpression promoted cell proliferation (Figure 2 C). Data from the immunoblot assays indicated that RCN2 overexpression increased CyclinD1 expression and RCN2 depletion decreased CyclinD1 expression in OSCC cells (Figure 2 D). Therefore, RCN2 promoted the proliferation of OSCC cells.

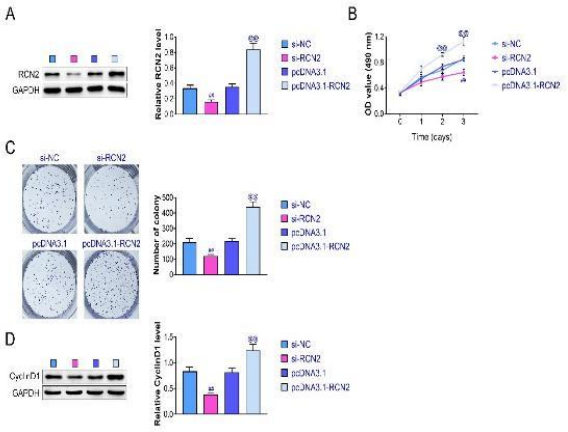

Figure 2: RCN2 promotes OSCC cell proliferation. (A). Expression of RCN2 in CAL-27 cells following plasmid or siRNA transfection. (B). Proliferation capacity of CAL-27 cells following plasmid or siRNA transfection. Absorbance at 490 nm was measured. (C). Proliferation capacity of CAL-27 cells following plasmid or siRNA transfection. (D) Expression of CyclinD1 in CAL-27 cells following plasmid or siRNA transfection. Data are presented as mean ± SEM, siRCN2 vs siNC, ## $p < 0.01$. pcDNA3.1-RCN2 vs pcDNA3.1, @@ $p < 0.01$. NC, negative control

RCN2 overexpression stimulated OSCC cell motility *in vitro*

Since RCN2 regulated the proliferation of OSCC cells, its effects on the motility of OSCC cells were examined through wound closure and transwell migration assays. RCN2 depletion significantly suppressed the migration of CAL-27 cells in the wound healing assay after 24 h, whereas RCN2 overexpression promoted wound healing. Thus, RCN2 overexpression stimulated the migration of OSCC cells (Figure 3 A). Subsequently, transwell assays revealed that RCN2 knockdown suppressed OSCC cell invasion, whereas overexpression of RCN2

promoted invasion (Figure 3 B). Therefore, overexpression of RCN2 also stimulated the motility of OSCC cells *in vitro*.

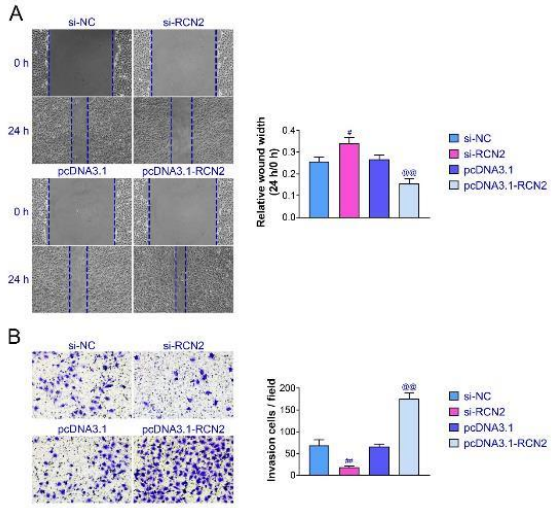

Figure 3: RCN2 stimulates the motility of OSCC cells *in vitro***.** (A) Migration capacity of CAL-27 cells following transfection with the indicated plasmids or siRNAs. Wound widths were measured after 24 h. (B) Invasion capacity of CAL-27 cells following transfection with the indicated plasmids or siRNAs. Migrated cells were counted. Data are presented as mean ± SEM, siRCN2 vs siNC, # $p < 0.05$, ## $p < 0.01$. pcDNA3.1-RCN2 vs pcDNA3.1, @@ $p < 0.01$. NC, negative control

RCN2 regulated the EGFR/ERK pathway in OSCC cells

The mechanism underlying RCN2 promotion of OSCC progression was investigated. A previous study indicated that the EGFR/ERK pathway mediates the proliferation and motility of OSCC cells and affects the progression of OSCC. Therefore, the effects of RCN2 on the EGFR/ERK pathway were examined using immunoblot assays. RCN2 depletion decreased the phosphorylation levels of EGFR and ERK, and its overexpression increased phosphorylation levels in CAL-27 cells (Figure 4 A). The EGFR inhibitor erlotinib was used. RCN2 overexpression promoted the proliferation of OSCC cells, whereas erlotinib treatment prevented the promotion of OSCC cell proliferation due to RCN2 overexpression (Figure 4 B). Similarly, the expression of CyclinD1 and the phosphorylation levels of ERK were reversed after erlotinib treatment in RCN2-overexpressing CAL-27 cells, further confirming our conclusion (Figure 4 C). Therefore, RCN2 mediated the EGFR/ERK pathway in OSCC cells, thus regulating the proliferation and motility of OSCC cells.

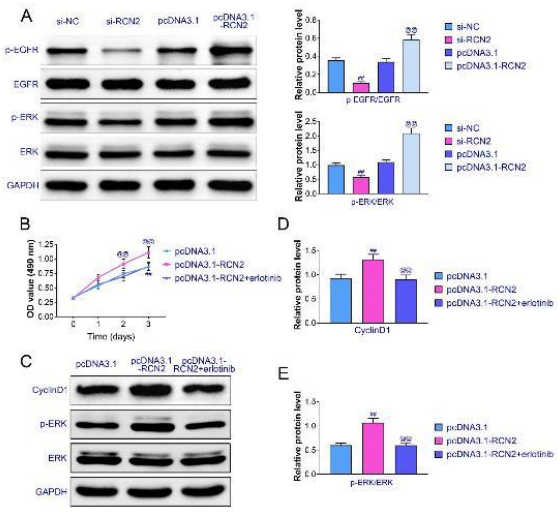

Figure 4: RCN2 mediated the EGFR/ERK pathway in OSCC cells. (A) Expression of p-EGFR, EGFR, p-ERK, and ERK in CAL-27 cells following transfection with the indicated plasmids or siRNAs. (B) Proliferation capacity of CAL-27 cells following the indicated treatments. (C - E) Expression of Cyclin D1, ERK, and p-ERK in CAL-27 cells following the indicated treatments. Data are presented as mean ± SEM; siRCN2 vs siNC, ## $p < 0.01$. pcDNA3.1-RCN2 vs pcDNA3.1, @@ $p < 0.01$. NC, negative control

DISCUSSION

OSCC is an important type of oral cancer with high morbidity and mortality. It is the most malignant and harmful tumor of the head and neck [10]. OSCC is prone to distant metastasis, such as lung, bone, lymphatic, and blood-borne metastases. Once distal metastasis occurs, conventional surgical resection, radiotherapy, and chemotherapy have negligible effects. Therefore, early diagnosis and precise treatment of OSCC are particularly important. Although there has been some progress made in targeted therapies for OSCC, to combat this disease at an advanced stage, more effective therapeutic targets are still required [11]. Interestingly, the reticulocalbin RCN2 is present at abnormally high levels in human OSCC cells and tissues as compared to normal cells and tissues [12]. The effect of RCN2 on OSCC cell proliferation and motility was confirmed in our study. Therefore, RCN2 may be a promising target for OSCC treatment.

In this study, the effects of RCN2 on OSCC cells were examined, including proliferation and migration. The present results suggest that RCN2 expression affects the progression of OSCC. Furthermore, we examined whether RCN2 inhibitors affected the proliferation and migration of OSCC cells and inhibited tumor

growth. Finally, we investigated the relevant molecular mechanisms of RCN2.

Through CCK-8 and colony formation assays, the effects of RCN2 on OSCC cell proliferation were revealed. Wound healing and transwell assays indicated that RCN2 stimulated the motility of OSCC cells. The effects of RCN2 in several types of cancers have been reported. RCN2 overexpression is correlated with the recurrence and prognosis of patients with colorectal cancer [13]. In addition, RCN2 enhances HCC cell proliferation by targeting the EGFR-ERK pathway. Similarly, our results indicated that RCN2 promoted the proliferation and motility of OSCC cells via this pathway. However, the precise molecular mechanism requires further study.

Other reticulocalbins also affect the progression and development of cancers [14]. RCN1 depletion increases the sensitivity of cells to Adriamycin in nasopharyngeal carcinoma and stimulates cell apoptosis [15]. In addition, overexpression of RCN1 correlates with the poor prognosis of patients with non-small cell lung cancer (NSCLC) [16]. RCN1 was identified as a potential tumor marker in renal cell carcinoma and affects the progression of prostate cancer [17]. These studies provide evidence that the RCN family of proteins is widely involved in tumor regulation; thus, the design and development of relevant RCN inhibitors have the potential to treat tumors. However, bioinformatic analyses suggest that only RCN2 affects OSCC.

EGFR is widely overexpressed in a variety of cancers, including oral cancer. Overactivation of EGFR induces multiple downstream cell signaling pathways contributing to cancer development [17]. Multiple proteins promote the progression and development of OSCC via the EGFR/ERK pathway [18]. For example, the androgen receptor promotes OSCC cell migration by increasing EGFR phosphorylation. Similarly, RCN2 overexpression promoted the migration of OSCC cells through the EGFR/ERK pathway. Another study showed that curcumin inhibits OSCC proliferation and invasion via the EGFR pathway. In this study, RCN2 promoted the proliferation and motility of OSCC cells via the EGFR/ERK pathway, further suggesting that this pathway may serve as a target for OSCC treatment.

CONCLUSION

The findings of this study indicate that RCN2 is overexpressed in OSCC tissues and cells. Furthermore, RCN2 overexpression promotes the proliferation and motility of OSCC cells through EGFR/ERK pathway.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Wending Gao and Feng Cai contributed equally to the work and should be considered co-first authors. Wending Gao and Feng Cai designed the study and supervised data collection, Xiaoli Jiang analyzed and interpreted the data, and Chengchao Fan prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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